Metabolic Interconversions of Different Forms of Vitamin B₆*

(Received for publication, November 15, 1973)

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SUMMARY

Tritium-labeled pyridoxamine, pyridoxal 5'-phosphate, and pyridoxine 5'-phosphate were prepared and administered intravenously to mice, and the distribution of isotope in liver and carcass between pyridoxine, pyridoxine 5'-phosphate, pyridoxal, pyridoxal 5'-phosphate, pyridoxamine, and pyridoxamine 5'-phosphate was determined at different times after administration. The vitamin B₆ forms were extracted from the tissues by perchloric acid and separated by ion exchange chromatography. The results strongly suggest that phosphorylation is the first step in the conversion of pyridoxamine as well as of pyridoxine to the active coenzyme form of the vitamin. After an initial period of equilibration, pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate accounted for more than 90% of recovered isotope in liver and for 80 to 90% of recovered isotope in carcass. The ratio between these compounds was about 2:1 in both liver and carcass. About 10% of the total isotope in carcass appeared as pyridoxal. The available experimental data obtained after administration of different forms of vitamin B₆ were found to fit a kinetic metabolic model which assumes an equilibration between pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate but essentially unidirectional reactions in other metabolic conversions.

Although there is an extensive literature concerning the function of vitamin B₆ as a coenzyme in different enzymic reactions, little is known about the metabolism of the different forms of the vitamin in vivo. Several enzymes involved in the transformations of the vitamin forms into each other have been partially purified from microbeological and animal sources (for a review see Ref. 1). Although studies of individual enzymes have been of great value in suggesting possible metabolic pathways, they should be supplemented with in vivo studies. We have previously reported on the conversion of tritium labeled pyridoxine into other forms of vitamin B₆ in mouse liver and carcass (2). Subsequently, Colombini and McCoy (3) have carried out a similar study with the use of ¹³C-labeled pyridoxine. We have now prepared tritium-labeled pyridoxamine, pyridoxine 5'-phosphate, and pyridoxal 5'-phosphate and studied their metabolism in mice. On the basis of the reported results we have also attempted to formulate a metabolic model for the interconversion of the different vitamin forms in liver.

EXPERIMENTAL PROCEDURE

Compounds—Pyridoxine hydrochloride and pyridoxal 5'-phosphate were purchased from E. Merck AG (Darmstadt, Germany) and pyridoxine 5'-phosphate from Calbiochem (Los Angeles, California). Pyridoxamine 5'-phosphate, pyridoxamine hydrochloride, and pyridoxal hydrochloride were gifts from Merck Sharp & Dohme ( Rahway, N.J.). [³⁵S]Pyridoxine, with a specific radioactivity of 1 Ci per g and sodium borohydride, with a specific radioactivity of 11.8 Ci per mole, were obtained from the Radiochemical Centre (Amersham, Bucks, England).

Tritium-labeled Pyridoxamine—Pyridoxamine hydrochloride was labeled by exposure to tritium gas according to Wilzbach (4). The solution of tritiated pyridoxamine hydrochloride in water was dark brown. An aliquot corresponding to about 45 mCi was put onto a column (0.3-mm diameter, 400-mm height) of the cation exchanger Dowex AG50W-X8 (200 to 400 mesh, formate form). The column was washed with water and then eluted with ammonium formate by a gradient procedure as described below. About 3% of the isotope emerged at the position of pyridoxamine. The fractions which contained pyridoxamine were combined, the solvent was evaporated, and the residue was then dissolved in water and applied onto a column (0.3-mm diameter, 150-mm height) of Dowex AG50W-X8 (200 to 400 mesh, H⁺-form). This column was washed with 50 ml of water and eluted with 2 M hydrochloric acid. About 90% of the isotope which had been applied emerged in a single peak at 230 to 300 ml of effluent. An aliquot of the material was applied onto a column (0.3-mm diameter, 400-mm height) of Dowex AG50W-X8 (200 to 400 mesh, formate form) together with 1 mg of authentic pyridoxamine. The column was eluted with an ammonium-formate gradient. All of the labeled material appeared together with pyridoxamine in a single peak. The specific radioactivity of the purified pyridoxamine was about 0.1 Ci per g.

Synthesis of Tritium-labeled Pyridoxine 5'-Phosphate and Pyridoxal 5'-Phosphate—With a few modifications a method described by Stock et al. (5) was used. Pyridoxal 5'-phosphate (25 mg) was suspended in 300 µl of distilled water and brought into solution by addition of a small amount of sodium bicarbonate. The pH was adjusted to 7.5 to 8.0 with sodium carbonate, using indicator paper. Tritium-labeled sodium borohydride (300 µg, 100 mCi) was added to the solution at 0°C. Mixing was continued at this temperature for 15 min and the solution left at room temperature for another 15 min. Unlabeled sodium borohydride (800 µg) was then added in order to complete the reaction. The solution was again cooled to 0°C and 300 µl of 11.6 M perchloric acid and 100 µl of an aqueous solution containing 130 mg of manganese sulfate and 82 mg of potassium permanganate per ml were added. The reaction mixture was stirred for 15 hours at -13°C in total darkness and then centrifuged. The supernatant was added to 4.0 ml of a 1 M potas-
sium bicarbonate solution at 0°. The pH was adjusted to 6.5 with
0.5 M hydrochloric acid and the solution was again centrifuged.
The supernatant was kept frozen at -20°C.
One milliliter of the supernatant was chromatographed on a
column (400-mm height, 8-mm diameter) of Dowex AG50W-X8
(200 to 400 mesh), which had been equilibrated with an 0.01 M
ammonium formate buffer, pH 3.2. Elution was performed with
200 ml of the same buffer. Five-milliliter fractions were collected
at a flow rate of about 0.5 ml per min, and the isotope content was
determined in each fraction. Three distinct peaks (A, B, and C)
were obtained. They were identified by rechromatography
together with reference compounds, by paper chromatography (6),
by hydrolysis and chromatographic identification of the cor-
responding unphosphorylated B forms (2), and by high voltage
electrophoresis (7). These analytical procedures revealed that
Peak C was identified with [3H]pyridoxine 5'-phosphate. Peak B was
composed of [3H]pyridoxal 5'-phosphate and another unidentified
compound. These two compounds were separated on a col-
umn (80-mm height, 12-mm diameter) of Dowex AG50W-X8 (200
to 400 mesh; H+ form) eluted with 0.1 M hydrochloric acid.
Immediately before use in the biological experiments, pyridoxine
5'-phosphate and pyridoxal 5'-phosphate were chromatographed
once again on columns of Dowex AG50W-X8. These columns were
eluted with ammonium formate buffers of increasing pH and con-
centration. The purity of the eluted material was checked by the
above mentioned procedures (Fig. 1). The specific radioactivity
of the pyridoxine 5'-phosphate was 3.6 Ci per g and that of pyri-
adoxal 5'-phosphate was 1.5 Ci per g. The pH of the solutions was
adjusted to about 7.0.
Tritium-labeled pyridoxine 5'-phosphate in its conversion to
pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate loses 50% of
the label; this has been corrected for when calculating the dis-
tribution of isotope between different B forms.
Administration of Tritium-labeled Pyridoxamine, Pyridoxine
5'-Phosphate, and Pyridoxal 5'-Phosphate—Mice of the NMR
strain, with a weight of 20 to 25 g, were used. They had been
kept on a commercial mouse diet (Harald Fors Co., Stockholm,
Sweden). Each mouse in a series was injected with about 10 /*g
of [3H]pyridoxine 5'-phosphate or [3H]pyridoxal 5'-phosphate or
with 148 pg of [3H]pyridoxamine. The compounds were dissolved
in 200 /ul of water and administered intravenously in a tail vein.
Preparation of Tissues—The animals were killed by decapitation
at different times after the administration of the labeled com-
ounds. The livers were immediately removed and homogenized
in about 10 ml of water in a VirTis 29 homogenizer (VirTis
Co., Inc., Gardiner, N.Y.). The skin and head were removed from the
carcass which was homogenized in about 15 ml of water. The
volume of each homogenate was measured and an equal volume of
2% perchloric acid solution was added. The precipitated proteins
were removed by centrifugation at 800 x g. The supernatant was
then adjusted to pH 4.3 (glass electrode) with 3 M hydroxide
solution and kept at +4°C overnight in the dark. The precipitate of potassium perchlorate was removed by filtration,
the solution was evaporated to dryness at a bath temperature
below 40°C, and the residue dissolved in 10 ml of water.
Chromatographic Procedures—In the experiments in which the
pyridoxamine was administered we used the chromatographic
procedure reported previously (2). A suitable aliquot of the solu-
tion was put onto a column (9-mm diameter, 400-mm height) of
Dowex AG50W-X8 (200 to 400 mesh) which had been equilibrated
with a 0.06 M ammonium formate buffer, pH 4.25. The column
was first eluted with 100 ml of the same buffer and then with 0.5
M ammonium formate buffer of increasing pH. To establish the
gradient, 100 ml of 0.3 M ammonium formate buffer, pH 4.25,
were placed in a closed mixing flask, and 500 ml of 0.5 M ammonium
formate buffer, pH 7.5, were placed in the reservoir. The eluate
was collected in 5-ml fractions at a flow rate of 0.5 ml per min.
Pyridoxine 5'-phosphate and pyridoxal 5'-phosphate which did not
separate completely by this procedure were determined on a
second column as pyridoxine and pyridoxal, respectively, after
hydrolysis of the first 10 fractions with 0.2 M sulfuric acid.
An improved technique which resulted in good separation of
pyridoxine 5'-phosphate and pyridoxal 5'-phosphate was de-
veloped in the course of this work (8) and was used in the later
experiments. In those experiments the extract from liver or
carcass was chromatographed on a 9-mm diameter column (400-mm
height) of Dowex AG50W-X8 (200 to 400 mesh) which had been
equilibrated with 0.01 M ammonium formate buffer, pH 3.2. Elu-
tion was first performed with 200 ml of this buffer after which a
gradient system was started. It consisted of 100 ml of 0.05 M
ammonium formate, pH 4.25, in a mixing bottle to which was
added 0.5 M ammonium formate, pH 7.5. Fractions with a volume
of 5 ml were collected at a flow rate of 0.5 ml per min.
Determination of Isotope—The isotope content of the chromato-
graphic fractions was determined in a liquid scintillation spec-
trometer (Tri-Carb model 3375, Packard Instrument Co., La
Grange, III). Of each chromatographic fraction, 0.4 ml was added
to 16 ml of a counting mixture of the following composition: 2,5-
diphenyloxazole (PPO); 1.4-bis(2-methyl-5-phenyloxazolyl)benzene
(dimethyl POPOP), 0.3 g; toluene, 1000 ml; and methyl Cellosolve, 600 ml. The counts were corrected for quench-
ing by addition of 0.1 ml of an aqueous solution of tritium-labeled
pyridoxine as an internal standard. The efficiency of the counting
procedure was about 95%. A sufficient number of counts to give a
statistical error of less than 5% was recorded for each sample.
Assay of Vitamin B; Activity—Vitamin B; in chromatographic
fractions was estimated with a microbiological method using Sac-
charomyces carlsbergensis ATCC 9080, strain 4229 (9). From each
chromatographic fraction, 1 ml was added to 1 ml of 0.2 M sulfuric
acid. The solution was hydrolyzed at 120°C in an autoclave for 1
hour. The pH was then adjusted to 5.0 to 5.2 with a potassium
citrate buffer at pH 12. To 1 ml of the hydrolyzate was added 1
ml of water and 3 ml of the basal medium. Each tube was closed
with a plug of cotton, provided with a glass rod for stirring, steri-
lized for 10 min at 100°C, and then cooled. Yeast suspension (0.5
ml) was added and the test tubes were placed in a shaker for 18
hours at 30°C. The absorbance was then read at 540 nm in a Beck-
man B spectrophotometer. A standard series pyridoxine solutions
was carried through the whole procedure.
Quantitative Estimation of Pyridoxine 6'-Phosphate and Pyri-
oxal 5'-Phosphate—For quantitative estimations in the synthesis
of pyridoxine 5'-phosphate and pyridoxal 5'-phosphate we used a
fluorometric method described by Contractor and Shane (10) for
pyridoxal 5'-phosphate; it could be used also for the determi-
nation of pyridoxine 5'-phosphate.

![Fig. 1. Chromatography on columns of Dowex AG50W-X8 (400-mm height, 9-mm diameter; 200 to 400 mesh) of purified [3H]pyridoxine 5'-phosphate (top) and [3H]pyridoxal 5'-phosphate (bottom). Each compound was chromatographed together with 1 mg of the same unlabeled compound. Radioactivity, C; absorbance at 295 nm, O.](http://www.jbc.org/)

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Due to the vitamins' sensitivity to light, preparation of tissues has been performed in rooms with shaded light. All chromatographic procedures have been done in dark rooms and every analytical step has been performed as rapidly as possible in order to avoid chemical degradation or decomposition of the compounds in vitro.

**RESULTS**

Liver—A series of figures shows the distribution of isotope in liver between the different forms of vitamin B₆ after the administration of labeled pyridoxamine (Fig. 2), pyridoxine 5'-phosphate (Fig. 3), and pyridoxal 5'-phosphate (Fig. 4). It is apparent from these figures that the metabolic transformations of the administered precursor occurred within the first 1 to 2 hours after the injection. After this time an equilibrium was reached in which pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate accounted for 60 to 70% and 20 to 30%, respectively, of the recovered amount of isotope. The amount of isotope recovered as pyridoxamine or pyridoxine 5'-phosphate was less than 10% of the total 1 hour after administration of these compounds. After the administration of pyridoxal 5'-phosphate there was a rapid rise in the labeled pyridoxamine 5'-phosphate during the 1st hour after the injection, but no significant change in the proportion between labeled pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate during the following 2 hours. In all experiments labeled pyridoxal was found during the experimental period, but it accounted for less than 10% of the total labeled material. One series of animals which had been injected with labeled pyridoxamine was followed for 8 days. There appeared to be an increase in the relative concentration of pyridoxamine 5'-phosphate during this period.

Carcass—Figs. 5, 6, and 7 show the distribution of isotope between the different vitamin forms in carcass after administration of pyridoxamine, pyridoxine 5'-phosphate, and pyridoxal 5'-phosphate. Again, the final equilibrium was very similar, irrespective of which precursor was administered and was attained within the first few hours after administration of the labeled vitamin. Significant amounts of isotope were recovered only in pyridoxal 5'-phosphate, pyridoxamine 5'-phosphate, and pyridoxal. A series of mice injected with labeled pyridoxamine demonstrated that the proportion between labeled pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate remained essentially unchanged during a period of 8 days.

**Fig. 2.** Distribution of isotope between the different forms of vitamin B₆ in mouse liver after the intravenous injection of 148 μg of [3H]pyridoxamine. At times indicated two mice were killed and a perchloric acid extract of the livers was fractionated by ion exchange chromatography. Pyridoxamine, △; pyridoxamine 5'-phosphate, □; pyridoxal, ○; pyridoxal 5'-phosphate, •.

**Fig. 3.** Distribution of isotope between the different forms of vitamin B₆ in liver during the first 24 hours after the intravenous injection of [3H]pyridoxine 5'-phosphate. At times indicated two mice were killed and a perchloric acid extract of the livers was fractionated by ion exchange chromatography. Pyridoxine 5'-phosphate, ■; pyridoxal 5'-phosphate, •; pyridoxamine 5'-phosphate, △; pyridoxine, □; pyridoxal, ○.

**Fig. 4.** Distribution of isotope between the different forms of vitamin B₆ in liver during the first 3 hours after the intravenous injection of [3H]pyridoxal 5'-phosphate. At times indicated two mice were killed and a perchloric acid extract of the livers was fractionated by ion exchange chromatography. Pyridoxal 5'-phosphate, •; pyridoxamine 5'-phosphate, △; pyridoxal, ○.

**Fig. 5.** Distribution of isotope between the different forms of vitamin B₆ in mouse carcass after the intravenous injection of 148 μg of [3H]pyridoxamine. At times indicated two mice were killed and a perchloric acid extract of the carcasses was fractionated by ion exchange chromatography. Pyridoxamine, △; pyridoxamine 5'-phosphate, △; pyridoxal, ○; pyridoxal 5'-phosphate, •.
Specific Radioactivity of Pyridoxal 5'-Phosphate and Pyridoxamine 5'-Phosphate after Administration of [3H]Pyridoxamine—

It was considered of interest to establish to what extent an isotope equilibrium had been reached between pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate by comparing the specific radioactivity of these compounds. This was done in the series of mice injected with [3H]pyridoxamine and followed for 8 days. The chromatographic fractions containing pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate were collected and assayed by a microbiological method. The results are given in Fig. 8. It is apparent from this figure that there was no significant difference in the specific radioactivity of pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate in liver and carcass and also that the two vitamin forms reached approximately the same specific radioactivity at the end of the experimental period.

Kinetic Model of Vitamin B₆ Interconversions in Liver—The results presented in this work and in an earlier study of the metabolism of labeled pyridoxine (2) may be summarized in Fig. 9, an illustration of the main metabolic interconversions of vitamin B₆. In this scheme, k₁ to k₅ are rate constants for the transformations between the different forms of the vitamin. By standard procedures it is possible to write sets of differential equations which describe the time course of labeling of the different compounds after introduction of labeled material at different steps in the reaction sequence. If one uses a fixed set of values for the rate constants one would then expect the experimental curves to fit the model irrespective of which form of the vitamin that had been administered. We have first estimated k values from the different experiments and then used a computer program and a trial and error procedure to obtain values for the rate constants which would fit all the available data. Fig. 10 shows experimental points and the theoretical curves which were obtained with the following values for the rate constants: k₁ = 0.07 min⁻¹, k₂ = 0.11 min⁻¹, k₃ = 0.03 min⁻¹, k₄ = 0.07 min⁻¹, and k₅ = 0.04 min⁻¹.

DISCUSSION

These studies of vitamin B₆ metabolism have all been performed with tritium-labeled compounds because we wanted to use vitamin forms with the highest possible specific radioactivity. It has been suggested that tritium-labeled pyridoxine prepared by an exchange procedure, for example, may contain the two antimetabolites 4-deoxypyridoxine and 5-deoxypyridoxine as impurities (11). Great care was taken to establish the radiochemical purity of the compounds used in this study and we have not observed any evidence for the presence of the above mentioned antimetabolites. It has also been suggested by Colombini and McCoy that the use of tritium-labeled forms of vitamin B₆ is not devoid of serious inconveniences particularly when applied to metabolic studies (3) because of the presence of metabolically and in some situations chemically labile tritium atoms. It is of interest in this context that the results obtained by these authors, who used ¹⁴C-labeled pyridoxine and followed the dis-
Fig. 10. Theoretical distribution of isotope between the different forms of vitamin B₆ in liver. The curves were based on the model given in Fig. 9 with the following k values: $k_1 = 0.002$ min⁻¹, $k_2 = 0.11$ min⁻¹, $k_3 = 0.025$ min⁻¹, $k_4 = 0.07$ min⁻¹, and $k_5 = 0.04$ min⁻¹. A, labeling pattern after the administration of pyridoxine (experimental points were taken from Ref. 2); B, labeling pattern after the administration of pyridoxamine; C, labeling pattern after the administration of pyridoxine 5' phosphate; D, labeling pattern after the administration of pyridoxal 5' phosphate. Pyridoxine, □; pyridoxine 5' phosphate, ■; pyridoxal, ○; pyridoxal 5' phosphate, ●; pyridoxamine, △; pyridoxamine 5' phosphate, ▲.

Distribution between vitamin B₆ forms in mice, are very similar to those previously obtained with tritium-labeled pyridoxine (2). We have recently determined the distribution of tritium in [H₈]pyridoxine (12) and demonstrated that approximately 20% of the label is located in the 4-hydroxymethyl group, i.e. in a metabolically active position. The position of tritium atoms in the labeled pyridoxamine has not been determined but it seems reasonable to assume that the distribution is similar in [H₈] pyridoxine and in [H₈]pyridoxamine prepared by the same procedure. The method described by Stock and co-workers (5) for the synthesis of tritium-labeled pyridoxal 5' phosphate yielded labeled pyridoxine 5' phosphate as a by-product which could be isolated in pure form. These compounds are specifically labeled in position 4 and appropriate corrections for metabolic loss had to be made.

Several vitamin B₆ forms are quite unstable, and in experiments of the type described in this report it is possible that some decomposition occurs since, for example, exposure to light cannot be completely avoided. It has however been our impression that this decomposition is not serious since in the many chromatographic separations of tissue extracts more than 90% of the labeled material has appeared at the position of the six vitamin forms.

Conversion of pyridoxine to pyridoxal 5' phosphate could occur by two possible pathways: (a) pyridoxine → pyridoxal 5' phosphate or, (b) pyridoxine → pyridoxal 5' phosphate → pyridoxal 5' phosphate. The steps in these transformations would be catalyzed by pyridoxine dehydrogenase (EC 1.1.1.65) (Reaction 1) and pyridoxal kinase (EC 2.7.1.35) (Reaction 2) or by pyridoxal kinase (Reaction 3) and pyridoxal 5' phosphate oxidase (EC 1.4.3.5) (Reaction 4). The oxidase which catalyzes oxidation of pyridoxine 5' phosphate or pyridoxamine 5' phosphate cannot use pyridoxine as a substrate but Reaction 1 could be catalyzed by a pyridoxine dehydrogenase (EC 1.1.1.65) Studies with a partially purified pyridoxine dehydrogenase from rabbit liver have indicated that the equilibrium is strongly in favor of pyridoxine (1). Our previous in vivo studies with labeled pyridoxine have also demonstrated a rapid conversion of pyridoxine to pyridoxine 5' phosphate. In the present study a rapid disappearance of labeled pyridoxine 5' phosphate in both liver and carcass was followed by appearance of labeled pyridoxal 5' phosphate. In liver only small amounts of labeled pyridoxine and pyridoxal could be seen, whereas in carcass significant amounts of these compounds appeared, particularly during the 1st hour after administration. The presence of labeled pyridoxal in carcass after administration of pyridoxine was noted in our previous studies (2) as well as by Colombini and McCoy (3) who, however, reported much higher figures for the relative labeling of pyridoxal in carcass.
These authors interpret their results as evidence for a significant formation of pyridoxal 5'-phosphate from pyridoxine via the intermediate formation of pyridoxal (Pathway a). The present data and earlier results (2) for the time course of labeling of vitamin B₆ after injection of different precursors demonstrate that the main conversion to pyridoxal 5'-phosphate occurs at the phosphorylated level. The enzyme which catalyzes this reaction, pyridoxamine 5'-phosphate: oxygen oxidoreductase (EC 1.4.3.5), has a higher affinity for pyridoxine 5'-phosphate than for pyridoxamine 5'-phosphate, and it has been suggested that it should more appropriately be called pyridoxine 5'-phosphate oxidase (1). Pyridoxal most likely originates from the action of phosphatases on pyridoxal 5'-phosphate. The experiments in which labeled pyridoxal 5'-phosphate was administered (Figs. 4 and 7) clearly demonstrated the presence of such phosphatases, particularly in carcass. It is known that the unphosphorylated forms of vitamin B₆ are more easily transported across cell membranes than the phosphorylated forms (13-15), and it is possible that these forms of the vitamin, mainly pyridoxal, formed by the action of phosphatases are transported and rephosphorylated in other organs. It is interesting that Contraert and Shane have reported higher concentrations of pyridoxal and pyridoxine than of pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate in blood (10). It has also been assumed that the main degradative pathway for vitamin B₆ is the formation of 4-pyridoxic acid from pyridoxal. Recently, however, the above authors have reported that 4-pyridoxic acid 5'-phosphate is a normal metabolite formed from pyridoxal 5'-phosphate (16). The formation of pyridoxal 5'-phosphate from pyridoxamine could occur at the unphosphorylated or at the phosphorylated level, i.e. with the intermediate formation of (a) pyridoxal, or (b) pyridoxamine 5'-phosphate. Pyridoxal could be formed from pyridoxamine by the action of pyridoxamine 5'-phosphate oxidase and by transamination catalyzed by pyridoxamine pyruvate transaminase, an enzyme which does not transaminate phosphorylated vitamin forms (17, 18). The affinity of other transaminases apoenzymes for unphosphorylated B₆ forms is very low and evidence from enzymic studies would indicate that the two last mentioned reactions should be of small importance in vivo (1). The rapid increase in labeled pyridoxamine 5'-phosphate and in labeled pyridoxal 5'-phosphate in both liver and carcass after administration of pyridoxamine strongly suggests that phosphorylation is the first reaction. Again, labeled pyridoxal appeared, particularly in carcass, in about the same proportion as in the experiments discussed above. The rate of appearance of labeling in pyridoxal 5'-phosphate was similar when pyridoxine and pyridoxamine were administered. The enzyme ATP:pyridoxal 5'-phosphotransferase (EC 2.7.1.35) is believed to catalyze the phosphorylation of both pyridoxine and pyridoxamine. The affinity of the rat enzyme in vitro is higher for pyridoxine than for pyridoxamine (19, 20).

In almost all of the experiments with labeled vitamin B₆ in mice we have observed that an equilibrium is reached between pyridoxamine 5'-phosphate and pyridoxal 5'-phosphate with a ratio between the two compounds of approximately 1:2. After the initial period these two compounds have accounted for more than 90% of the recovered activity in liver and for 80 to 90% in carcass. The isotope ratio also represents the ratio between the mass concentration of these compounds since both present data (Fig. 8), and earlier results (2) indicate that there is no large difference in specific radioactivity between the different forms of vitamin B₆ in liver and carcass. Only limited information is available on the concentration of the different forms of vitamin B₆ in tissues. As early as 1948 Rabinowitz and Snell (21) who used a microbiological method reported 7.8 µg per g, wet weight, of rat liver for pyridoxal plus pyridoxal 5'-phosphate and 2.5 µg per g for pyridoxamine plus pyridoxamine 5'-phosphate. Later Bain and Williams (22) reported similar figures (pyridoxal 5'-phosphate, 6.0 µg per g, wet weight, and pyridoxamine 5'-phosphate, 3.2 µg per g, wet weight). In a detailed study Lyon and co-workers (23) found the ratio between pyridoxamine 5'-phosphate and pyridoxal 5'-phosphate to be 1.26 and 0.51 in liver and 0.15 and 0.29 in muscle in two different strains of mice fed the same stock diet, indicating that in the same species, genetic differences may determine the relative tissue concentrations of the individual forms of vitamin B₆.

Previous in vivo studies revealed a low fractional turnover rate for vitamin B₆ in rats 1.2 to 1.5% per day (24), and in man 2.2 to 4.4% per day (25). The data for specific radioactivity presented in Fig. 8 also indicate a low turnover rate. The complex network of vitamin B₆ metabolic reactions does not lend itself easily to a kinetic analysis. In this and earlier studies (2) labeled precursors have been introduced in the metabolic chain at different points which makes it possible to estimate the rate constants from several sets of experimental data. The scheme presented in Fig. 9 contains several simplifications. Thus, the action of phosphatases has been disregarded and elimination from the system has not been taken into account. To validate the model we used a single set of values for the rate constants to draw the theoretical curves for isotope distribution which would be expected after the administration of four different vitamin forms: pyridoxine, pyridoxine 5'-phosphate, pyridoxal 5'-phosphate, and pyridoxamine. These curves were compared with the experimental data. The fit was in no way perfect but it appears to us that the result (Fig. 10) shows that the scheme in Fig. 9 illustrates the main reactions in the metabolic transformation of vitamin B₆ and that the rate constants are correct in the order of magnitude, i.e. the rate constants for the metabolic transformations between different forms of the vitamin are at least 1000 times higher than the rate constants for elimination from the body.

It should be observed that we have presented figures for the distribution of isotope calculated as per cent of recovered amount of isotope in the different forms of vitamin B₆. Only in the case of pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate is there an appreciable steady state physiological pool, and only for these compounds is it therefore possible to give approximate figures for the mass conversion. From the literature (26) approximate figures for pyridoxal 5'-phosphate and pyridoxamine 5'-phosphate would be 4 and 5 µg per g of mouse liver, i.e. the total interconversion between these two compounds would amount to about 0.2 to 0.3 µg per min.

If one considers the whole animal the situation becomes even more complex than in liver and no attempt was made to make any quantitative estimates of rate constants. One would have had to take into account the fact that the fractional turnover rate of total vitamin B₆ is different in different organs and tissues (15, 27), and that the relative proportion of the different forms may vary. The results from measurements of transformations in carcass do, however, indicate that in general equilibrium is rapidly attained and that labeling occurs in the same sequential order as in liver.

The approach used in this paper may be of value in attempts to explain abnormalities in the metabolism of vitamin B₆. For instance, Lyon and co-workers (23, 28) and Bell and Haskell (20, 30) have published extensive studies in a strain of mice, I/St,
which is acutely sensitive to vitamin B₆ depletion and susceptible to audiogenic seizures. In this strain there is a high ratio of pyridoxamine 5'-phosphate to pyridoxal 5'-phosphate in brain and liver, most marked on a B₆ deficient diet. There is also higher excretion of 4-pyridoxic acid than in control animals, but a low concentration of the enzyme which converts pyridoxal to 4-pyridoxic acid, aldehyde:oxygen oxidoreductase (EC 1.2.3.1). Also, the $K_m$ value of pyridoxamine 5'-phosphate oxidase for pyridoxamine 5'-phosphate and for pyridoxine 5'-phosphate is 4 times higher than in control animals. Apparently, however, B₆-deficient diets result in the same degree of depletion in animals of the I/St strain and in control animals. It would be of interest to establish if the observed differences in concentration of active forms of the vitamin and the enzyme differences are reflected in vivo in changes in rates of the metabolic interconversions in the whole animals or in specific organs, e.g. in the brain.

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